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Spatially Dependent Dynamic MAPK Modulation by the Nde1-Lis1-Brap Complex Patterns Mammalian CNS

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SUMMARY

Regulating cell proliferation and differentiation in CNS development requires both extraordinary complexity and precision. Neural progenitors receive graded overlapping signals from midline signaling centers, yet each makes a unique cell fate decision in a spatiotemporally restricted pattern. The Nde1-Lis1 complex regulates individualized cell fate decisions based on the geographical location with respect to the midline. While cells distant from the midline fail to self-renew in the Nde1-Lis1 double-mutant CNS, cells embedded in the signaling centers showed marked overproliferation. A direct interaction between Lis1 and Brap, a mitogen-activated protein kinase (MAPK) signaling threshold modulator, mediates this differential response to mitogenic signal gradients. Nde1-Lis1 deficiency resulted in a spatially dependent alteration of MAPK scaffold Ksr and hyperactivation of MAPK. Epistasis analyses supported synergistic Brap and Lis1 functions. These results suggest that a molecular complex composed of Nde1, Lis1, and Brap regulates the dynamic MAPK signaling threshold in a spatially dependent fashion.

INTRODUCTION

The developmental formation of the mammalian CNS is controlled by precise spatial and temporal integration of mitogenic and neurogenic signals. These signals collectively guide neural progenitors to divide and differentiate into structurally and functionally diversified neurons and their supporting cells. While CNS development follows the general rules of organogenesis in that cell division and differentiation are coordinated in space and time to generate tissue of the proper size, shape, and cytoarchitecture, the cell fate decisions of neural progenitors are governed by signal molecules secreted from specialized cells localized in embryonic signaling centers (Chizhikov and Millen, 2005; Jessell, 2000; Megason and McMahon, 2002; Wilson and Maden, 2005). The major signaling centers in the developing CNS are located along the midline throughout the anterior-posterior (AP) axis of the neural tube. These midline-associated structures provide mitogenic and neurogenic signals that reach neural progenitors in the form of gradients and exert functions in a spatially dependent manner (Gurdon and Bourillot, 2001). In this topology, the cell fate decision of neural progenitors, to a large extent, is determined by the geographic position or distance from the signaling center. Progenitors that are close to the signaling centers receive signals at a higher concentration, whereas progenitors that are distant from the signaling center sense reduced signals and may have to alter the signaling sensitivity and threshold for decision making. However, the cell signaling mechanism by which neural progenitors respond differentially to graded signaling molecules, faithfully convert them to binary fate decisions between proliferative self-renewal and neuronal differentiation, and ultimately generate a widely divergent variety of neurons in defined regions of the mammalian CNS is not well understood.

The traditional understanding of signal transduction that guides cell division and differentiation is largely based on pathway maps, which include linear sequences of ordered activation of cellular events, such as protein phosphorylation, and transcriptional enhancement or suppression of gene expression. The pathway maps have become more complex; increasing examples of pathway crosstalk have turned them into intricate signaling networks. Nonetheless, the number of signal pathways and molecules are limited in comparison to the vastly divergent yet specific cell fate decisions of neural progenitors. This suggests that each individual progenitor, depending on its respective location to the signaling center, may use a specific mechanism for individualized interpretation of mitogenic and morphogenic signals.

The concept of individualized cell signaling dynamics was first demonstrated in studies of mitogen-activated protein kinase (MAPK) pathway activation in PC12 cells, where transient activation of the MAPK cascade led to proliferation but sustained and ultrasensitive MAPK activation resulted in neuronal differentiation (Marshall, 1995). The three-tiered MAPK pathway,

composed of Raf, mitogen-activated protein kinase kinase (MEK), and extracellular signal-related kinase (ERK), is known to act as a critical regulator for various physiological signaling inputs and to serve as a relay route from the cell surface to the nucleus. The MAPK cascade enables cells to interpret external signals and to respond in an appropriate way to control bistable switches of cell fate decisions such as proliferation, differentiation, migration, senescence, and apoptosis (Wellbrock et al., 2004). The ability to convert analog signals into switch-like all-or-none cell fate decisions by the MAPK module is believed to be accomplished by scaffold proteins that bind to multiple components of the MAPK pathway (Brown and Sacks, 2009; Grewal et al., 2006). These scaffold proteins may enable the use of the MAPK cascade in a highly context-dependent manner, leading to diversified signaling outputs. The major scaffolds that facilitate MAPK activation in metazoans are known to be KSR (kinase suppressor of Ras) and its family members (Brennan et al., 2011; McKay et al., 2009; Morrison, 2001). By interacting with Raf, MEK, and ERK, KSR facilitates Ras-dependent activation of Raf and the MAPK cascade. It is subsequently phosphorylated by ERK and serves as a signal attenuator by releasing the MAPKs from the plasma membrane (Clapéron and Therrien, 2007). Although the MAPK signaling cascade has been shown to play an important role in controlling cell proliferation and differentiation in the developing CNS, the scaffold protein(s) and molecular mechanisms responsible for converting graded signals from the midline signaling centers to self-renewal or neuronal differentiation in each class of neural progenitors have not yet been identified.

The *NDE1* gene is an essential player in CNS neurogenesis. Its recessive mutations result in profound malformation of the entire CNS, while the non-CNS organs are, in contrast, relatively well developed (Alkuraya et al., 2011; Bakircioglu et al., 2011). In addition to extreme microcephaly (small brain) and lissencephaly (smooth brain), patients carrying *NDE1* mutations often show cranial facial midline-related dysmorphisms and expansion of the choroid plexus (CP). Nde1 was initially discovered through its high-affinity binding to LIS1 (Feng et al., 2000), encoded by a gene in which haploinsufficiency results in lissencephaly. Both Nde1 and Lis1 are versatile and multifaceted molecules that synergistically participate in the dynamic formation of multiple protein complexes to maintain progenitor self-renewal and radial migration of cortical neurons in the developing cerebral cortex. In a model of extreme Nde1-Lis1 double deficiency ($Nde1^{-/-}Lis1^{+/-}$), the mutant brain was less than 20% of the normal brain in size with remarkable loss of neocortical neurons and their lamination. This was largely due to the failure of the mutant radial glial cells to self-renew at the onset of cortical neurogenesis, resulting in precocious neurogenesis and programmed cell death. Loss of basal-lateral membrane stability also led to aberrant neuronal migration (Pawlisz and Feng, 2011; Pawlisz et al., 2008).

In the present study, we extend the previous findings of the neocortical specific function of the Nde1-Lis1 complex to broader spatially dependent cell fate modulation in the developing CNS. We report that besides the loss of neocortical neurons, Nde1-Lis1 double-deficient mice showed remarkable reduction of dorsal spinal cord somatosensory neurons. The severe neuronal loss paradoxically coexisted with the overproliferation of cells along the dorsal midline. Such preferential loss of neocortical and dorsal spinal cord neurons and gain of midline epithelial cells correlated with altered MAPK signaling was mediated by a direct interaction between the Nde1-Lis1 complex with Brap, a Ras effector, negative regulator of KSR, and threshold modulator for the MAPK cascade (Li et al., 1998; Matheny et al., 2004). Consistent with a role in MAPK modulation, the Nde1-Lis1 mutant showed an altered response to reduced epidermal growth factor (EGF) and fibroblast growth factor (FGF), as well as elevated Ksr, phospho-Mek (pMek), and phospho-Erk (pErk) specifically in the medial CNS. Mouse genetic analysis also illustrated a functional synergistic partnership among Nde1, Lis1, and Brap. These data collectively suggested that a molecular complex including Nde1, Lis1, and Brap can modulate the MAPK scaffold in a dynamic, spatially dependent fashion. Such regulatory mechanism may fine-tune the way mitogenic signals are interpreted in progenitors depending on their position relative to the midline, allowing graded extracellular inputs to generate diversified cell fate decisions.

RESULTS

Spatial Dependence of Nde1-Lis1 Function in the Developing CNS

Similar to patients who carry recessive *NDE1* mutations, the Nde1-Lis1 double-deficient mice ($Nde1^{-/-}Lis1^{+/-}$) showed neurogenesis and morphogenesis defects in the entire CNS (Figure 1; Figure S1 available online) (Pawlisz et al., 2008). In addition to the profound loss of neocortical neurons (Figures 1A and 1B), remarkable neuronal reduction was observed in the dorsal spinal cord of the $Nde1^{-/-}Lis1^{+/-}$ mutant (Figures 1D–1G). We found that a large number of dorsal sensory interneurons were missing (Figures 1D–1F). Pan-neuronal marker NeuN, interneuron marker GABA, and interneuron subtype markers Calretinin and Calbindin all collectively indicated near-complete loss of interneurons in the dorsal horn of the mutant, whereas the neuronal loss in the ventral spinal cord of the mutant was relatively modest (Figures 1E and 1G). In contrast to the severe malformation of the CNS, the size and shape of the dorsal root ganglia (DRG), which are derived from the neural crest, were indistinguishable from those of the controls (Figure 1E). These observations, combined with our previous reports of preferential loss of neocortical neurons in the $Nde1^{-/-}$, $Nde1^{+/-}Lis1^{+/-}$, and $Nde1^{-/-}Lis1^{+/-}$ mutants (Feng and Walsh, 2004; Pawlisz et al., 2008), demonstrate that the functions of Nde1 and Lis1 are more essential in cells of the dorsal CNS.

In addition to the marked loss of neurons in the neocortex and the dorsal spinal cord, the $Nde1^{-/-}Lis1^{+/-}$ mutant was also characterized by the widening of dorsal-midline-associated structures. The roof plate, which is known to secrete and supply mitogenic and morphogenic signals to neural progenitors in the dorsal aspect, was significantly expanded (Figures 1D and 1E). Rostrally, the mutant brain was characterized by the massive expansion of the CP epithelium cells (Figures 1B and 1C). Although the loss of neurons was expected by the well-recognized requirement of Nde1 and Lis1 in neural progenitor mitosis, the paradoxical expansion of the dorsal midline structures was surprising and noninterpretable by previously identified molecular functions of Nde1 and Lis1. Thus, these observations

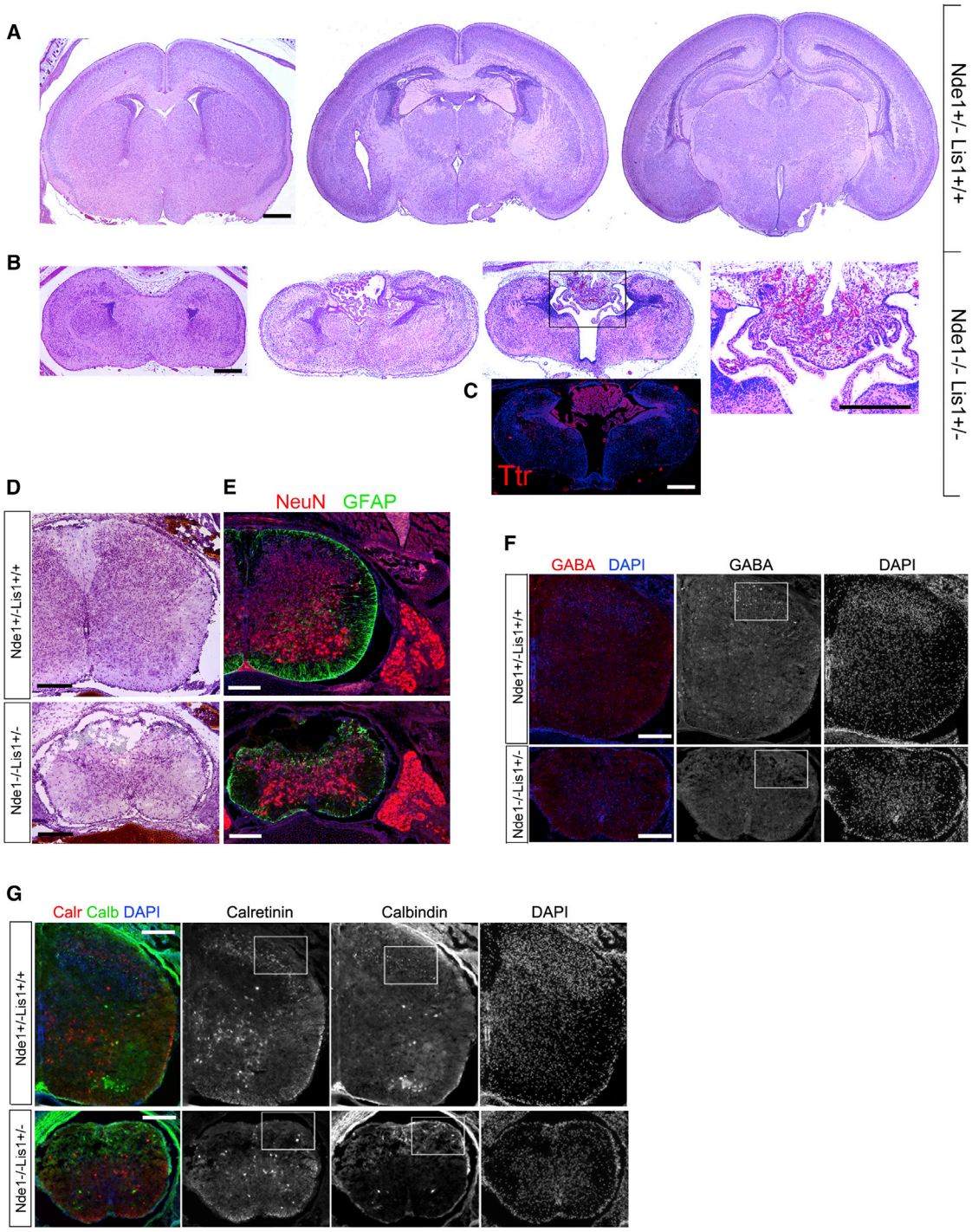

Figure 1. Spatially Dependent CNS Phenotypes of Nde1-Lis1-Deficient Mice

(A and B) Hematoxylin and eosin staining of the brain of Nde1$^{-/-}$Lis1$^{+/-}$ mutants and their Nde1$^{+/-}$Lis1$^{+/+}$ littermates at birth (P0).

(C) Transthyretin (Ttr) IH analysis of CP epithelium.

(D) Nissl staining of the spinal cord of the Nde1$^{-/-}$Lis1$^{+/-}$ mutant and its Nde1$^{+/-}$Lis1$^{+/+}$ littermate at E18.5.

(E) Double IH staining of neurons (NeuN) and astrocytes (GFAP) in the spinal cord of a Nde1$^{-/-}$Lis1$^{+/-}$ mutant and its Nde1$^{+/-}$Lis1$^{+/+}$ littermate at E18.5.

(F and G) IH analyses of interneurons in the spinal cord of a Nde1$^{-/-}$Lis1$^{+/-}$ mutant and its Nde1$^{+/-}$Lis1$^{+/+}$ littermate at E18.5.

Bars represent 200 μm. See also Figure S1.

suggested that the mechanism by which the Nde1-Lis1 complex regulates cell proliferation and differentiation is much more complex than those revealed in the current literature and that Nde1 and Lis1 may function differentially in different cell groups.

Loss of Progenitors and Neurons Most Distal to the Midline Signaling Centers

To gain additional insight into the cell-type-dependent function of Nde1 and Lis1 in CNS development, we examined different classes of neurons and/or neural progenitors in the Nde1-Lis1 double-mutant spinal cord. In the developing spinal cord, distinct classes of neurons are generated in discrete domains along the dorsal-ventral (DV) axis from similarly organized progenitor domains. Neurogenesis is controlled by signaling molecules secreted from opposing signaling centers on the ventral and dorsal midline called floor plate (FP) and roof plate (RP), respectively. These signaling molecules act as mitogens and/or morphogens, providing positional information to neural progenitors, and guide the spinal cord to develop into domains of distinctive cell types at defined positions along the DV axis. The generation of motor neurons in the ventral spinal cord, which occurs during embryonic day (E) 10 to 11 in mice, is largely governed by graded Sonic Hedgehog (Shh) secreted from the FP and its underlying notochord. The Shh gradient patterns the ventral spinal cord and controls the expression of several cross-repressive homeodomain transcriptional factors, including Nkx2.2 and Nkx6.1 (Jessell, 2000). Although neuronal loss was widespread throughout the entire CNS, we found that the Nkx2.2+ and Nkx6.1+ domains were largely intact in the Nde1$^{-/-}$Lis1$^{+/-}$ mutant (Figures 2A and S2), suggesting that patterning of the ventral spinal cord by Shh was not altered significantly. Likewise, the dorsal-most domain of the ventral spinal cord marked by Pax6 was only moderately affected by the mutation (Figure 2A). In line with the well-preserved ventral neural progenitors, relatively subtle reduction of Isl1+ motor neurons and V2 interneurons was observed in the mutant (Figures 2B, 2C, and S2).

In contrast to the moderate phenotype in ventral domains, striking defects were observed in the dorsal spinal cord. Dorsal progenitors defined by Pax7 or Pax3 expression were greatly reduced in the mutant (Figures 2D and 2E, boxes; Figures 2F and S2). Although the loss of Pax3+ progenitors was remarkable in regions distal to the RP, Pax3+ RP cells and neighboring progenitors did not show significant loss (Figure 2E). Since the Nde1$^{-/-}$Lis1$^{+/-}$ mutation appeared to affect progenitors in a position-specific manner, we analyzed the regional distribution of neural progenitors by equally dividing the spinal cord along the DV axis into five bins (where bin 1 and bin 2 correspond to the dorsal portion and bin 4 and bin 5 the ventral portion) and analyzed the fraction of progenitors in each bin using the pan-progenitor marker Sox2. This analysis showed that the most severe loss of progenitors in the Nde1$^{+/-}$Lis1$^{+/-}$ mutant occurred in bin 2 (Figure 2H). This region is distant to both dorsal and ventral midline signaling centers and approximately overlaps with the DI2–DI3 progenitor domain (Figure 2G, brackets; Figure 2H). As the DI2 progenitors are known to express Ngn1, we examined Ngn1 expression in the mutant by in situ hybridization (ISH). As expected, Nde1-Lis1 double deficiency resulted in a significant loss of Ngn1+ progenitors in the dorsal spinal cord, whereas Ngn1 expression was only slightly reduced in the ventral aspect of spinal cord and not altered in the DRG in the mutant (Figure 2I). While the Isl1+ DI3 neurons were present in the dorsal aspect, they were reduced and disorganized in the mutant (Figure 2B, higher-magnification inserts; Figure 2C). Thus, these data together suggested that loss of Nde1-Lis1 function most strongly impaired progenitors in a region that largely overlaps with the DI2/DI3 class of progenitors from which the sensory neurons in the dorsal horn are derived. The severe progenitor loss in the dorsal spinal cord resembled what we have found previously in the mutant's neocortex, where more progenitors left the cell cycle precociously, resulting in the elimination of aberrantly formed neurons by apoptosis (Figures 2J and S2; Pawlisz et al., 2008). The increase in the cell-cycle withdrawal in the mutant was also found to be spatially dependent, affecting bin 2 most severely (Figure 2J). Therefore, the severe loss of progenitors in the DI2/DI3 domain resulted from reduced self-renewal of progenitors positioned distantly from both the RP and the FP.

Overproliferation of Midline-Associated Cells

Contrary to marked neural progenitor loss distal to the midline, the Nde1$^{-/-}$Lis1$^{+/-}$ embryos showed overproliferation of cells associated with the dorsal midline throughout the entire AP axis of the neural tube. Starting from E11, ISH analyses with the RP markers Msx1 and Lmx1a demonstrated a significantly expanded RP in the Nde1$^{-/-}$Lis1$^{+/-}$ spinal cord (Figure 3A). Cells in the expanded RP continued to divide through E13 and often resulted in the folding of RP epithelium cells and the formation of a "roof tube" on top of the neural progenitors and newborn neurons (Figures 3B and 3C).

In the developing telencephalon, the medial-most domain equivalent to the RP is composed of precursors to the CP. These nonneural epithelial cells produce cerebrospinal fluid and their proliferation is controlled by BMPs that drive the expression of Msx1. Enlarged Msx1+ domains were detected in the Nde1$^{-/-}$Lis1$^{+/-}$ brain as early as E12.5, which correlated with the onset of cortical neurogenesis. The expansion of Msx1+ cells became more evident after E13 (Figure 3D), and these cells appeared to be mature CP epitheliums, as evidenced by the expression of Transthyretin (Ttr) and other epithelium markers (Figures 3E and S3). In contrast to the expansion of the RP and the CP, their immediately adjacent domains (i.e., the DI1 domain in the spinal cord and the cortical hem in the telencephalon, marked by Math1 and Wnt2b expression, respectively) were not altered significantly in the Nde1$^{-/-}$Lis1$^{+/-}$ mutant (Figures 3A and 3D). Taken together, the cell proliferation phenotype caused by Nde1-Lis1 double deficiency appeared to be distinctively correlated with the location of the cell and its distance from the midline signaling center. Neural progenitors distant from the midline in the neocortex and the spinal cord DI2/DI3 domains failed to self-renew, whereas cells embedded in the signaling center in the CP and RP overproliferate. Progenitors situated between these two domains in the cortical hem and in the DI1 domain appeared unaltered. Thus, the function of Nde1-Lis1 complex is spatially dependent in the developing CNS; such spatial dependence with respect to midline signaling centers may reflect differential regulatory mechanisms to graded mitogens or morphogens.

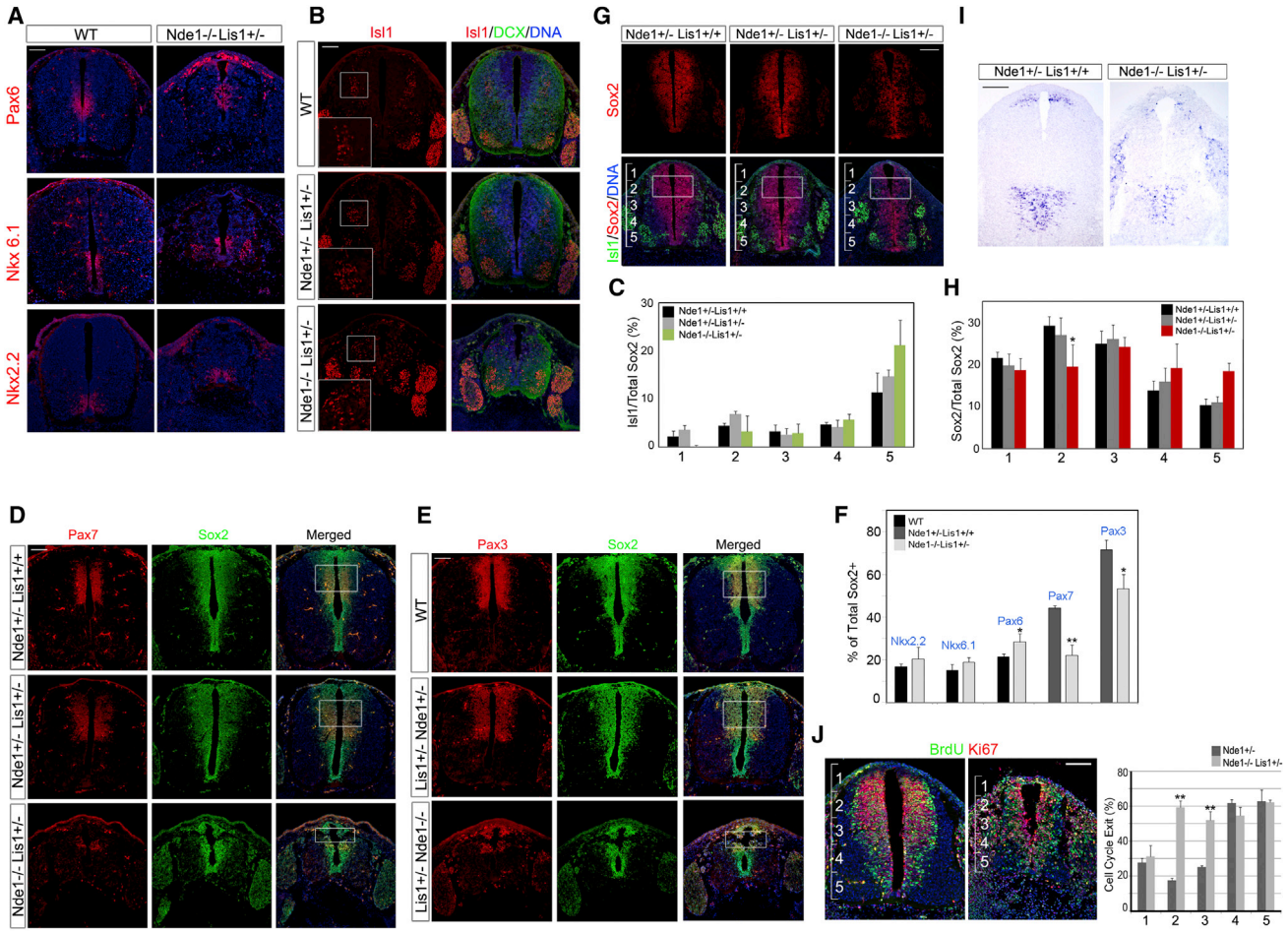

Figure 2. Specific Loss of the DI2/DI3 Class of Neural Progenitors in the Developing Spinal Cord of Nde1$^{-/-}$Lis1$^{+/-}$ Mice

(A) IH analysis with markers for ventral (Nkx2.2 and Nkx6.1) and middorsal (Pax6) progenitors at E12.5.

(B) IH analysis with pan-neuronal marker DCX and motor and DI3 neuron marker Isl1 at E12.5. Higher-magnification inserts of Isl1 immunosignals indicate the loss and disorganization of the DI3 neurons in the Nde1$^{-/-}$Lis1$^{+/-}$ mutant.

(C) Distribution of Isl1+ neurons along the DV axis (mean +SD from four litters). The spinal cord was equally divided into five bins from RP to FP. Isl1+ neurons in each bin were counted and normalized to total progenitors marked by Sox2.

(D and E) IH analysis with the dorsal progenitor markers Pax7 and Pax3 and the pan-progenitor marker Sox2 at E12.5. Boxed areas indicate the severe loss of progenitors.

(F) Percentage of each neural progenitor subclass over total Sox2+ progenitors (mean +SD from four litters) in the developing spinal cord of Nde1$^{-/-}$Lis1$^{+/-}$ mutants and their wild-type or Nde1$^{+/-}$Lis1$^{+/+}$ littermates at E12.5.

(G) IH analysis with Sox2 and Isl1 in Nde1$^{-/-}$Lis1$^{+/-}$ and control littermates at E11.5. Boxed areas indicate the severe loss of progenitors.

(H) Distribution of neural progenitors along the DV axis (mean +SD from more than three litters). The spinal cord was equally divided into bins 1–5 from RF to FP. Sox2+ progenitors in each bin were counted and normalized to total Sox2+ progenitors.

(I) ISH analysis of Ngn1+ progenitors at E11.5.

(J) The Nde1$^{-/-}$Lis1$^{+/-}$ mutants and their littermates were pulse labeled by bromodeoxyuridine (BrdU) at E10.8 and analyzed at E11.5 by double IH labeling with BrdU (green) and Ki67 (red) antibodies. Fractions of cells in each of the five bins (described in C and H) that were BrdU+ but Ki67− (green) over total BrdU-labeled cells (green + yellow) are presented as mean +SD.

*$p < 0.03$, **$p < 0.001$ by Student's t test. Bars represent 100 μm. See also Figure S2.

Altered Sensitivities to Reduced Growth Factor Concentration

To test the hypothesis that the spatial dependence of Nde1-Lis1 function is via modulating a cell's response to the graded morphogens and/or mitogens, we isolated neural progenitors from the cerebral cortex of Nde1$^{-/-}$Lis1$^{+/-}$ mutants and their littermates at E12.5 and studied them in culture. As the reduction of neural progenitors in the Nde1$^{-/-}$Lis1$^{+/-}$ cortex was already substantial at E12.5 and over half of the remaining progenitors were leaving the cell cycle (Pawlisz et al., 2008), it took longer (~2 weeks) to establish neurosphere cultures from the Nde1$^{-/-}$Lis1$^{+/-}$ progenitors than from cells isolated from the control littermate (3–5 days). However, once established, the Nde1$^{-/-}$Lis1$^{+/-}$ culture could be maintained over a period of several months by dissociating and diluting every 3–5 days. While the growth rate of Nde1$^{-/-}$Lis1$^{+/-}$ progenitors was lower

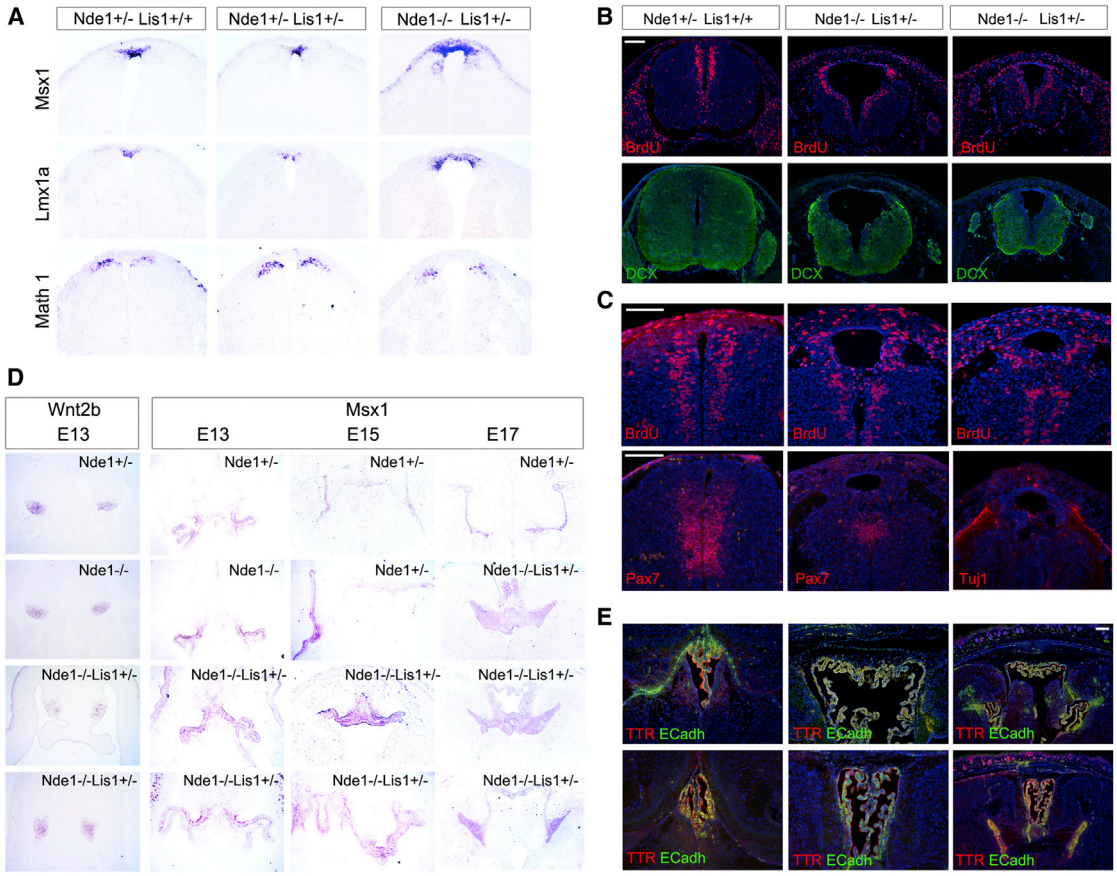

Figure 3. Expansion of the RP and CP due to Nde1-Lis1 Deficiency

(A) ISH analyses with markers for the RP (Msx1 and Lmx1a) and DI1 progenitors (Math1) of spinal cords at E11.5.

(B) Co-IH analysis of neurons (DCX) and S phase progenitors by 30 min BrdU labeling at E12.5.

(C) IH analysis of the developing spinal cord at E12.5. Note that overproliferation of the RP in the Nde1$^{-/-}$Lis1$^{+/-}$ mutant results in the formation of a tubular structure (roof tube).

(D) ISH studies of the developing telencephalon with CP and BMP signaling indicator Msx1 and cortical hem marker Wnt2b.

(E) IH analysis with the CP marker TTR and epithelium marker E-cadherin at birth (P0).

Bars represent 100 μm. See also Figure S3.

than control cells, very few Nde1$^{-/-}$Lis1$^{+/-}$ progenitors showed spontaneous neuronal differentiation or apoptosis during the extended culture period, which was in great contrast to their diminished self-renewal in vivo. Similar to control lines, neuronal differentiation of the cultured Nde1$^{-/-}$Lis1$^{+/-}$ progenitors could be induced by growth factor withdrawal, producing healthy neurons and astroglial cells with morphologies essentially indistinguishable from the control lines derived from the wild-type littermates (Figure 4A) (Pawlisz and Feng, 2011).

The discrepancy in proliferation and differentiation of Nde1$^{-/-}$Lis1$^{+/-}$ progenitors in vivo and in culture suggested that Nde1 and Lis1 conduct a function that is specific to the developmental tissue context and that such function is altered in the culture environment. The standard neurosphere culture media contains 20 ng/ml EGF and 10 ng/ml FGF (Reynolds and Weiss, 1992). This concentration of growth factors is relatively high compared to physiological conditions. This rich culture condition may resemble the environment in regions near the midline, such as cells in the DI1 region and the cortical hem, so that the growth and self-renewal disadvantages caused by Nde1$^{-/-}$Lis1$^{+/-}$ mutation may be compensated for by the higher concentration of mitogens. Since we hypothesized that the Nde1-Lis1 deficiency might result in an altered response to mitogens in the mutant, leading to increased proliferation under high mitogenic signals but poor proliferation in tissues where mitogenic signals were low, we experimentally reduced the concentration of growth factors in the culture medium to mimic the environment of neural progenitors in regions distant from the midline signaling centers. When we reduced EGF and FGF in the neurosphere culture media by 10-fold, we found that Nde1$^{-/-}$Lis1$^{+/-}$ neurospheres became less tight and misshapen; they turned into "neurosheets" in 48 hr (Figure 4B), which better mimicked their phenotype in vivo (Pawlisz and Feng, 2011). After being cultured in reduced growth factors, progenitors were then plated on Matrigel for 24 hr to examine cell differentiation. A significantly higher fraction of Nde1$^{-/-}$Lis1$^{+/-}$ progenitors was found to have undergone neuronal/glial differentiation and became TuJ1/DCX+ neurons or GFAP+ astrocytes

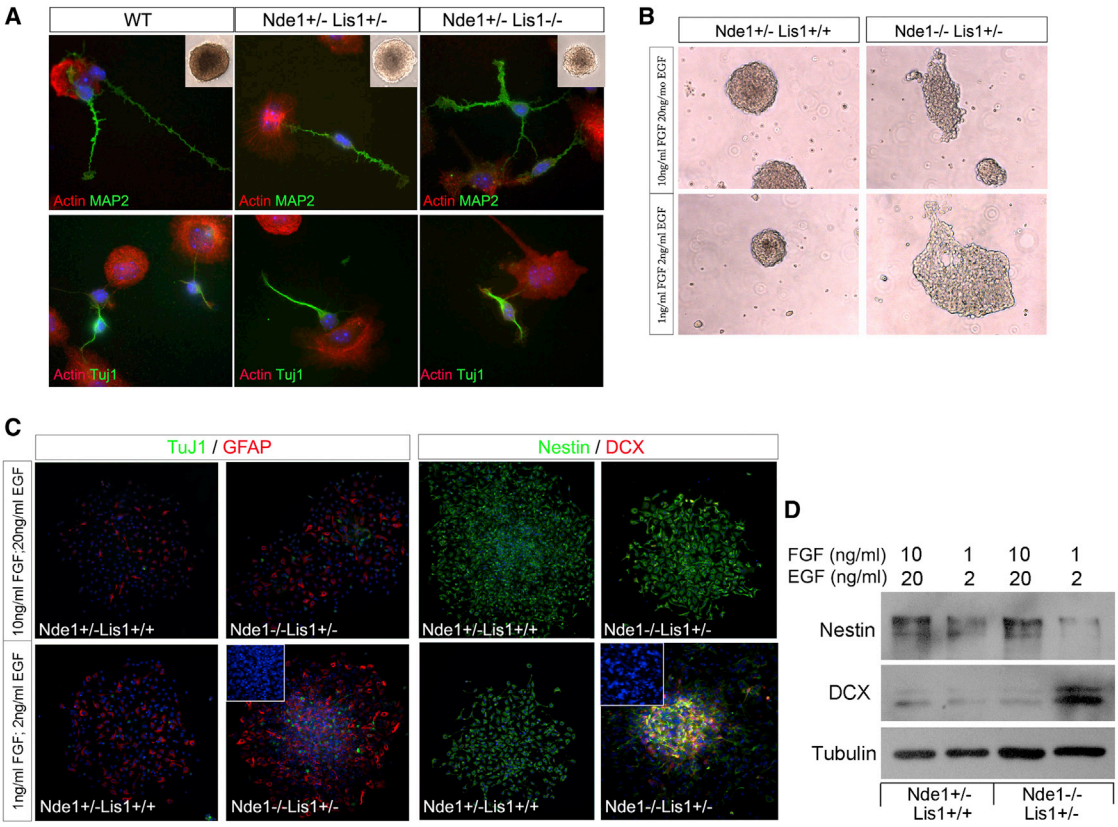

Figure 4. Altered Dependence to Growth Factor Concentrations of Nde1-Lis1-Deficient Neural Progenitors

(A) The Nde1$^{-/-}$Lis1$^{+/-}$ mutant progenitors form nearly normal neurospheres under standard culture conditions with 20 ng/ml EGF and 10 ng/ml FGF. Upon growth factor withdrawal, they differentiated into neurons and glial cells with normal morphology and express MAP2 and Tuj1 in 48 to 72 hr. Cells were also stained with rhodamine-phalloidin to visualize F-actin.

(B) After culturing in medium of reduced EGF and FGF (2 ng/ml EGF and 1 ng/ml FGF) for 48 hr, the cell-cell adhesion defect of Nde1$^{-/-}$Lis1$^{+/-}$ mutant progenitors was worsened.

(C) After being cultured in medium with reduced growth factors, the Nde1$^{-/-}$Lis1$^{+/-}$ mutant and control neurospheres were analyzed by immunofluorescence with the progenitor marker nestin, neuron markers DCX or Tuj1, and glial marker GFAP. Increased apoptosis, viewed by Hoechst staining (blue) of condensed nuclei, is shown in higher-magnification inserts.

(D) Reduced nestin coupled with increased DCX was detected by western blotting in Nde1$^{-/-}$Lis1$^{+/-}$ neurospheres after being cultured in medium with reduced EGF and FGF for 24 hr.

(Figure 4C). Significantly reduced nestin coupled with increased DCX was also observed in Nde1$^{-/-}$Lis1$^{+/-}$ cells on immunoblots, but the control Nde1$^{+/-}$Lis1$^{+/+}$ cells cultured under the same condition retained the profiles of neural progenitors (Figure 4D). These data showed that the Nde1$^{-/-}$Lis1$^{+/-}$ cells had altered sensitivity to EGF/FGF and confirmed a mitogen-concentration-dependent cell fate defect of the Nde1$^{-/-}$Lis1$^{+/-}$ progenitors. This suggests that the Nde1-Lis1 complex may play a role in sensing the concentration of mitogens and converting the graded midline signals to a position-dependent binary cell fate decision between self-renewal and neuronal differentiation.

Association to the MAPK Module through Interaction with Brap

The spatially divergent cell proliferation phenotype in different regions of the Nde1-Lis1-deficient CNS and the altered cell fate in response to reduced EGF and FGF of the Nde1$^{-/-}$Lis1$^{+/-}$ mutant neural progenitors led us to consider a possible mechanism in which Nde1, Lis1, and their associated protein complex(es) act as cell signaling adaptors in regulating the differential conversion of the graded midline signals. Both Lis1 and Nde1 are scaffold proteins known to exist in multiprotein complexes in various cellular compartments. Besides interacting with the cytoskeleton and maintaining mechanical features of the neural progenitors, they can also interact with other molecules. From the LIS1 yeast two-hybrid screen in which Nde1 was discovered (Feng et al., 2000), multiple clones that encode Brap were also pulled out. Brap was originally identified as a BRCA1 (BR) associated (A) protein (P) (Li et al., 1998). It was subsequently found to conduct a stimulus-induced binding to the GTP-loaded Ras, inactivate the MAPK scaffold protein KSR, and negatively regulate ERK1/2 activation by uncoupling Raf kinase from MEK. Moreover, Brap is an E3 ubiquitin ligase that undergoes autoubiquitination upon Ras activation, which releases Brap's repression of KSR and the MAPK cascade (Matheny et al., 2004). Such dual-effector regulation by Ras through Brap may limit the assembly of the MAPK component, restrict signal propagation, set signaling thresholds, and prevent unnecessary activities

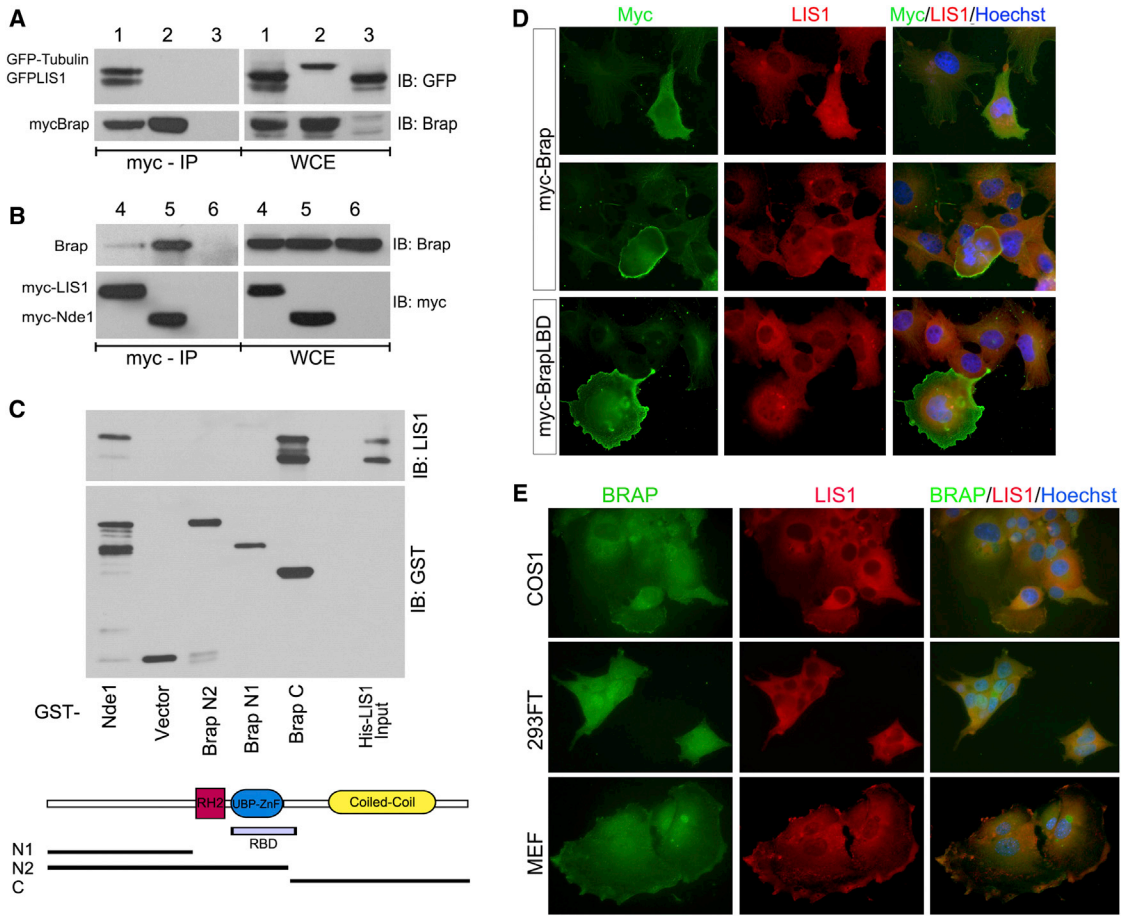

Figure 5. Interaction of the Nde1-Lis1 Complex with Brap

(A and B) Coimmunoprecipitation analyses of Brap with the Nde1-Lis1 complex. Recombinant myc- or EGFP-tagged Brap was coexpressed with myc- or EGFP-tagged LIS1, Nde1, or tubulin in 293T cells. Immunoprecipitations were performed with the c-Myc 9E10 monoclonal antibody followed by immunoblotting for EGFP-tagged proteins as indicated. Lane 1: EGFP-LIS1 + mycBrap; lane 2: EGFP-tubulin + mycBrap; lane 3: EGFP-LIS1; lane 4: EGFP-Brap + mycLIS1; lane 5: EGFP-Brap + mycNde1; lane 6: EGFP-Brap.

(C) GST pull-down of His-LIS1 with GST-Brap fusion constructs. Three GST-Brap fusion constructs were expressed, purified from bacteria on glutathione-agarose, and incubated with bacterial recombinant His-LIS1. Their binding to His-LIS1 was analyzed by immunoblotting with a LIS1 antibody, with GST-Nde1 as the positive control. The structure of Brap and the GST-Brap fusion constructs used for the analysis are depicted. RBD, Ras binding domain.

(D) Immunofluorescence costaining of endogenous LIS1 and transfected myc-Brap or myc-LBD in COS1 cells.

(E) Immunofluorescence analysis of LIS1and Brap in MEFs, 293T, and COS1 cells. Notice the invisible nuclei in Brap-immunostained cells, which suggest the cell surface association with Brap.

See also Figure S4.

in response to extracellular stimuli. Therefore, the interaction of Lis1 with Brap supports the notion that Nde1 and Lis1 may be part of the molecular complex required for regulating a previously unrecognized, mitogen induced MAPK signal transduction in neural progenitors.

To test the hypothesis that the Nde1-Lis1 complex coordinates with Brap in regulating the MAPK signaling, we first confirmed the physical interaction between Lis1 and Brap. Myc- or EGFP-tagged Brap was generated and coexpressed with LIS1 and Nde1 in 293T cells for coimmunoprecipitation analyses. While GFP-LIS1 was found specifically in the immunocomplex of mycBrap, Brap could also be detected conversely in the immunoprecipitate of mycLIS1 (Figures 5A and 5B). Although Brap was not found as a protein that interacts directly with Nde1 through yeast two-hybrid analyses (Figure S4), a significant amount of Brap was observed in the immunoprecipitate of mycNde1 (Figure 5B). As Nde1 was known to interact with LIS1 with very high affinity and the recombinant mycNde1 efficiently complexes with endogenous LIS1, these data suggested that recombinant Brap interacts with native LIS1 more efficiently than with recombinant GFP- or Myc-tagged LIS1. By binding to LIS1 simultaneously, Brap and Nde1 may have overlapping functions and act in the same molecular complex.

The direct interaction between Brap and Lis1 was further confirmed by glutathione S-transferase (GST) pull-down assays. The 594-amino-acid Brap protein contains multiple domains, including an N-terminal domain, a ring finger (RING-H2) domain predicted to have E3 ubiquitin-protein ligase activity flanked by an ubiquitin protease-like zinc finger (UBP-ZnF), and a C-terminal coiled-coil domain that resembles structural maintenance of

chromosomes (SMC) proteins. To identify the domain by which Brap interacts with Lis1, we made several bacterial GST fusion proteins encoding the N-terminal domain, with or without the RING-H2 and UBP-ZnF structures, and the C-terminal domain of Brap, and tested their direct interactions with bacterial recombinant His-LIS1. While the C terminus of Brap showed a strong interaction with LIS1, neither the N terminus of Brap nor a larger construct that includes the domain involved in ubiquitin E3 activity showed detectable LIS1 binding (Figure 5C). Since the previously reported minimal Ras binding domain largely overlaps with the UBP-ZnF but not in the C terminus (Matheny et al., 2004; Matheny and White, 2006), our data indicate that Brap interacts with LIS1 directly with its C terminus coiled-coil. Such interaction should not directly block the function of Brap as an E3 ubiquitin-protein ligase, protease, or a Ras effector in MAPK signaling regulation.

Although the interaction of recombinant Brap with endogenous LIS1 was mainly observed in the cytoplasm, a significant fraction of Brap was detected to colocalize with LIS1 near the cell surface by immunofluorescence (Figure 5D). The yeast two-hybrid assay further narrowed down the Lis1 binding domain of Brap to amino acid S453 through Q544 (Figure S4). A greater fraction of this Lis1-binding domain (LBD) appeared to be cell-surface associated when expressed in COS1 cells (Figure 5D). To confirm the association of an Nde1-Lis1-Brap complex with the plasma membrane, we generated several antibodies against Brap. The Brap antibody recognized Brap specifically (Figure S4); it highlighted the cell peripheries and showed colocalization with LIS1 in multiple cell types (Figure 5E). Together, these data supported the notion that the Brap-Lis1-Nde1 complex may be localized beneath the plasma membrane where they mediate the regulation of the MAPK signal transduction cascade from the cell surface to cytoplasm.

Spatially Dependent MAPK Activation in the Developing CNS

The MAPK cascade is a central signaling relay for cell proliferation, differentiation, survival, and apoptosis. The interaction of Lis1 and Nde1 with Brap thus suggests a regulatory mechanism for MAPK signaling modulation in neural progenitors. To test the functional interaction among Lis1-Nde1, Brap, and the Brap-associated MAPK cascade, we examined the MAPK activity in the Lis1-Nde1 mutant embryos. Significantly elevated phospho-Erk1/2 (pErk) was detected in the Nde1$^{-/-}$Lis1$^{+/-}$ brain (Figure 6A), indicating either increased strength and/or duration of the MAPK pathway signaling. Correlated with the higher level of pErk, increased Ksr was also observed in the mutant brain (Figure 6B), suggesting that the high level of MAPK activity in Nde1$^{-/-}$Lis1$^{+/-}$ brain was a result of enhanced engagement of the MAPK module through elevating its scaffold Ksr. This supports a model in which Nde1 and Lis1, together with Brap and Ksr, act in concert in a multimolecular complex that regulates the dynamic assembly of MAPK. Further examinations of the phospho-MAPK distribution demonstrated that the elevated MAPK activity was spatially dependent in the Nde1$^{-/-}$Lis1$^{+/-}$ mutant. We dissected the brain of Nde1$^{-/-}$Lis1$^{+/-}$ mutant embryos and their littermates into medial and lateral parts to compare their levels of phospho-MAPK by immunoblotting and found that both pErk and pMek were significantly increased in the medial brain of the mutant. The alteration of MAPK activity was correlated with the Ksr levels in both medial and lateral brain. While the medial brain of the mutant showed simultaneous elevation of pMek, pErk, and Ksr, the lateral brain of the mutant did not show these changes, but rather showed a decrease in Ksr (Figure 6C). These demonstrated that Nde1 and Lis1 regulate MAPK signaling differentially in the medial and lateral brains, and they suggested that Nde1-Lis1 is an integral part of a spatially dependent dynamic modulator of the MAPK assembly and may act as a "buffer" system to control the proper level of Ksr.

Similar findings in spatially dependent MAPK regulation by Nde1 and Lis1 were shown by immunohistologic analysis. In the developing spinal cord, more intense pErk immunosignals were detected in both the FP and RP of the Nde1$^{-/-}$Lis1$^{+/-}$ mutant (Figure 6D). Likewise, elevated pErk was found in the midline and signaling centers of the developing forebrain. At E11.5, the early onset of cortical neurogenesis, the pErk signals in the dorsal midline domains including CP and cortical hem precursors were equally strong in both the mutant and controls (Figure S5). However, at the peak of CP growth and cortical neurogenesis from E14 to E17, stronger pErk signals became obvious in cells of the mutant midline, including the CP epithelial cells and cortical hem neuroepitheliums (Figures 6E and 6F). These support the notion that, through the interaction with Brap and the differential regulation of Ksr, the Nde1-Lis1 complex buffers the thresholds for the MAPK activation, so that the activity is suppressed by raising the threshold in cells bombarded by a high concentration of mitogenic signals in the midline signaling center but reducing the threshold and sensitizing the mitogenic response in neocortical and Dl2/Dl3 neural progenitors that are distant from the midline signals, so that these progenitors may undergo extended self-renewal and generate more neurons.

Synergistic Functions of Brap with Lis1 in CNS Development

To further verify the functional interaction between Nde1-Lis1 and Brap, we generated Brap knockout mice. Brap is a conserved gene across eukaryotes with a single ortholog present in each species, and it shows broad tissue expression in mammals (Li et al., 1998). Gene targeting in mouse embryonic stem (ES) cells was performed to delete exon 2 and 3 of the murine Brap gene, which is predicted to result in a nonsense truncation mutation after amino acid A27 (Figure 7A). Analyzing a large number of Brap mutant mice and embryos indicated that the Brap homozygous mutant (Brap$^{-/-}$) was able to survive to E12 with approximately the predicted Mendelian inheritance but die in the following 24 hr before E13 (Table S1). Few homozygous-mutant embryos showed significant difference in size or morphology from their wild-type littermates (Figure 7B).

To confirm the previously reported role of Brap in regulating MAPK dynamic signaling, we isolated mouse embryonic fibroblasts (MEFs) from the Brap$^{-/-}$ embryos and examined their MAPK responses to growth factors in culture. The Brap$^{-/-}$ MEFs showed a higher amplitude of Erk phosphorylation than the wild-type MEFs upon FGF stimulation (Figures 7C and S6). Not only was the MAPK response to FGF stronger in the Brap$^{-/-}$ MEFs, but the mutant MEFs also had increased sensitivity to

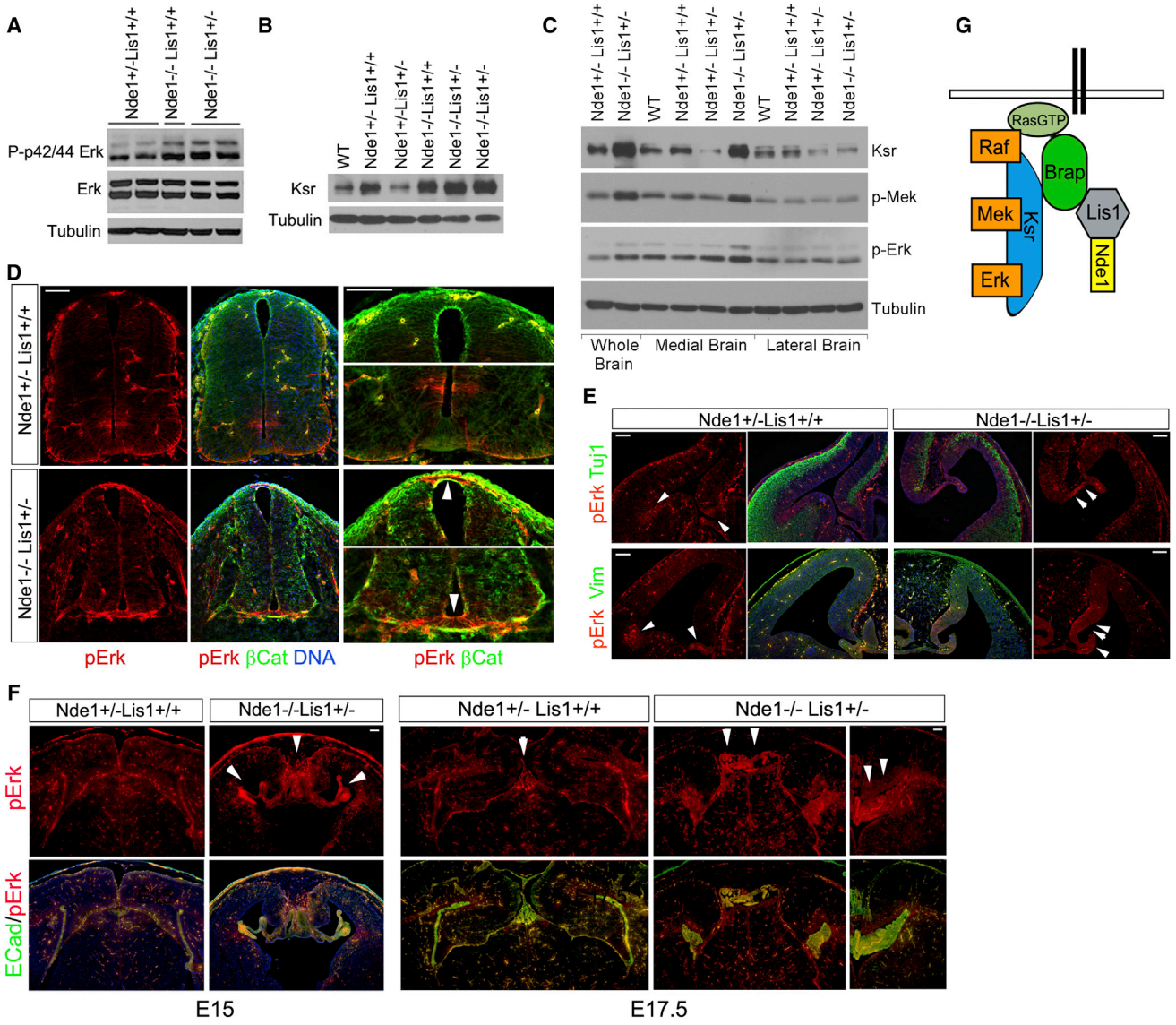

Figure 6. Spatially Dependent Alteration of MAPK Activity in Nde1-Lis1-Deficient Mice

(A and B) Immunoblotting of total protein extracts from the cerebral cortex of Nde1$^{-/-}$Lis1$^{+/-}$ embryos and their littermates at E12.5. More than three litters were analyzed, and data from representative litters with two or more Nde1$^{-/-}$Lis1$^{+/-}$ embryos were chosen for presentation.

(C) Medial-lateral distribution of MAPK activities in the Nde1$^{-/-}$Lis1$^{+/-}$ brain and their littermates. Whole brains were dissected from E12.5 embryos, divided into medial and lateral parts, and flash frozen in liquid N_2. Total proteins were extracted in hot SDS sample buffer and analyzed by immunoblotting with antibodies indicated.

(D) IH analyses of pErk distribution in the spinal cord of Nde1$^{-/-}$Lis1$^{+/-}$ mutant and its control littermate at E11.5. Arrows indicate intense pErk signals in the RP and FP of the mutant. The sections were counterstained with β-catenin and Hoechst for nuclei DNA.

(E) IH analysis of pErk distribution in the developing forebrain at E14. Arrows indicate elevated pErk. The sections were costained with the pan-neuronal marker Tuj1 and radial glial marker vimentin, respectively, along with Hoechst stain for nuclei DNA.

(F) IH analyses of pErk distribution in the brain of Nde1$^{-/-}$Lis1$^{+/-}$ embryos and their littermates at E15 and E17.5. Enhanced pErk signals (arrows) were observed in the excessively overproliferated CP epithelium cells. Sections were coimmunostained with the epithelial marker E-cadherin. Bars represent 100 μm.

(G) A diagram to illustrate molecular relationships between Nde1, Lis1, Brap and the MAPK signaling cascade. Note that the molecular interactions may be dynamically regulated in a spatially specific manner in the developing CNS.

See also Figure S5.

FGF over the wild-type. More robust Erk phosphorylation could be induced by a low dose of FGF (Figure 7D). Similar to Brap$^{-/-}$ MEFs, MEFs isolated from the Nde1$^{-/-}$Lis1$^{+/-}$ mutant showed alteration in the amplitude and duration of MAPK signaling in response to FGF (Figure S6). Consistent with the altered MAPK dynamics in culture, Brap$^{-/-}$ mutant brains showed elevated pErk and Ksr (Figure 7C), which was reminiscent of what we observed in the Nde1$^{-/-}$Lis1$^{+/-}$ mutant.

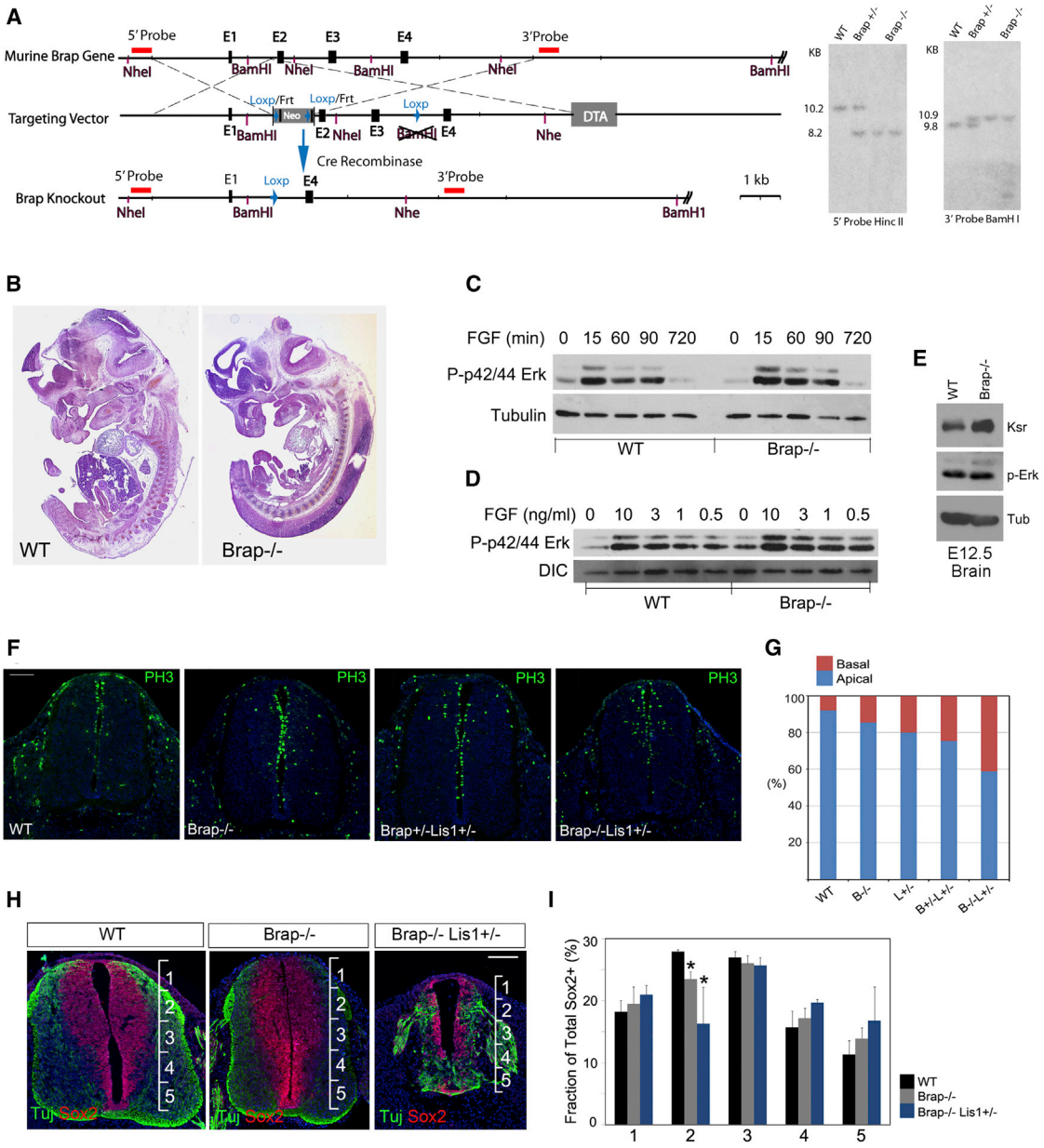

Figure 7. Synergistic Interaction of Brap and Lis1

(A) Murine Brap gene knockout strategy and Southern blot analyses used to confirm the Brap$^{+/-}$ and Brap$^{-/-}$ mutations.

(B) Nissl staining of a Brap$^{-/-}$ embryo and its wild-type littermate at E12.5.

(C and D) Immunoblotting of MEFs isolated from wild-type and Brap$^{-/-}$ embryos. Cells at passage 2 or 3 were serum starved for 40–44 hr and then treated with 10 ng/ml recombinant FGF or as indicated.

(E) Immunoblotting of total proteins extracted from the brains of E12.5 Brap$^{-/-}$ embryos and wild-type littermates.

(F and G) IH analysis of the Brap and Lis1 double-mutant embryos at E11.5 with the metaphase progenitor marker PH3. Over 200 PH3+ metaphase cells from three litters were counted. The percentage of cells with apically metaphase PH3 signals in each genotype is presented.

(H and I) IH analysis of neural progenitor distribution in Brap$^{-/-}$Lis1$^{+/-}$ mutants and wild-type and Brap$^{-/-}$ littermates. Sox2+ neural progenitors were colabeled with the neuron marker Tuj1. Fractions of Sox2+ progenitors (mean +SD of three litters) are presented as described in Figure 2H.

*$p < 0.03$ by Student's t test. Bars represent 100 μm. See also Figure S6.

The early embryonic lethality of Brap$^{-/-}$ mutants precluded us from detailed analysis of Brap's role in cerebral cortical development, but it provided an opportunity to delineate the molecular and functional relationship between Lis1 and Brap in spinal cord development by performing epistasis analysis. The Lis1$^{-/-}$ mutant was peri-implantational lethal; the Lis1$^{+/-}$ mutant showed normal body size but relatively mild defects in CNS development (Hirotsune et al., 1998). One of the clearly documented phenotypes of the Lis1$^{+/-}$ (and Nde1$^{-/-}$) embryos is that the mutant neural progenitors are not able to precisely

position the metaphase chromosomes to the apical surface through a process known as interkinetic nuclear migration (INM). We therefore examined INM by labeling the metaphase chromatin with the phosphohistone H3 antibody (PH3). In the wild-type neural progenitors, over 92% of the metaphase chromosomes were found at the apical lumen along the neural tube; a subtle defect in INM was shown by the Brap$^{-/-}$ embryos, but over 85% of the metaphase chromosomes were still correctly localized at the apical surface. A total of 80% of the Lis1$^{+/-}$ progenitors was found normal in this test, which was comparable to the previous published results (Gambello et al., 2003). However, reducing the levels of Brap in the Lis1$^{+/-}$ embryos resulted in significant dosage-dependent synergistic phenotypes; the INM defect was more pronounced in Brap$^{+/-}$ Lis1$^{+/-}$ than in Lis1$^{+/-}$ embryos, and it was further worsened in Brap$^{-/-}$Lis1$^{+/-}$ mutant, in which less than 60% of the metaphase neural progenitors were able to move their nuclei toward the apical lumen (Figures 7F and 7G). This dosage-sensitive INM defect of Lis1 and Brap mutants suggests that the two proteins act in concert, forming a molecular complex to regulate the cell-cycle-dependent nuclear movement in neural progenitors.

Besides the INM defect, many Brap$^{-/-}$Lis1$^{+/-}$ mutants showed significant reductions in size and became lethal around E11.5 (Table S1). Although the early lethality made it difficult to analyze the phenotype in dorsal midline expansion, which is most significant after E11.5 in the Nde1$^{-/-}$Lis1$^{+/-}$ mutant, some Brap$^{-/-}$Lis1$^{+/-}$ embryos showed widening of the RP at E11.5. Also similar to the Nde1$^{-/-}$Lis1$^{+/-}$ mutant, the Brap$^{-/-}$Lis1$^{+/-}$ mutant showed a spatially dependent reduction of progenitors distal to the RP and FP. We analyzed the distribution of Sox2+ neural progenitors as described in Figure 2H. While Brap$^{-/-}$ mutants showed a mild reduction of Sox2+ progenitors in Bin 2, the Brap$^{-/-}$Lis1$^{+/-}$ mutant showed a profound progenitor loss in this bin (Figures 7H and 7I). Taken together, these genetic epistasis studies demonstrated the dosage-sensitive close partnership between Brap and Lis1 in neural progenitors. The Brap$^{-/-}$ Lis1$^{+/-}$ mutation phenocopies Nde1$^{-/-}$Lis1$^{+/-}$, indicating that Nde1, Lis1, and Brap participate synergistically in a common molecular interactome to modulate the spatially dependent sensitivity and threshold of MAPK signaling in CNS development.

As a scaffold protein, Lis1 is fundamental to cell survival through its interactions with many cellular proteins other than Nde1 and Brap. To ensure that phenotypes observed in Nde1, Lis1, and Brap mutants were directly mediated by a dosage-dependent function of Lis1 but not secondary effects through alteration of other Lis1 binding proteins, we examined the level and cellular localization of eight known Lis1 binding proteins as well as cell organelle positioning and focal adhesion in Brap$^{-/-}$ and Nde1$^{-/-}$Lis1$^{+/-}$ mutant brains and/or MEFs but found no significant changes (Figure S6). These data, plus the fact that non-CNS organs were largely unaffected in the Brap$^{-/-}$ and Nde1$^{-/-}$Lis1$^{+/-}$ mutants, argue that the spatially dependent function of Nde1, Lis1, and Brap in neural progenitors is cell and cell-context specific.

DISCUSSION

In this study, we identified a mechanism by which graded signals emanating from the midline of the embryonic CNS are differentially interpreted by a protein complex involving Lis1, Nde1, and Brap through modulation of MAPK dynamics. This regulatory mechanism allows each individual cell to react in a position-dependent manner to multiple overlapping mitogens and morphogens and to interpret them over a wide concentration range to make precise cell fate decisions about proliferation, self-renewal, and neuronal differentiation. Malfunctions in this cell-signaling module hampered self-renewal of progenitors situated far away from the signaling center where mitogen concentration is low but led to overproliferation of cells closer to or inside the signaling centers where mitogen concentration is at the peak level. Thus, our results provide insight into the molecular basis for a spatially dependent individualized cell fate decision. They also help to explain how a limited number of signaling molecules may generate a much greater variety of outputs for cell proliferation and differentiation in mammalian CNS development.

Morphogen and Mitogen Gradients and Cell Fate Decisions

In the developing CNS, mitogenic/morphogenic molecules from location-specific signaling centers form counteracting gradients and simultaneously guide orchestrated cell proliferation, neuronal differentiation, and tissue morphogenesis (Edlund and Jessell, 1999). It is well recognized that multiple midline signal molecules such as Bmp, Wnt, Fgf, and Shh can each dictate the expression of a set of genes responsible for cell-type specification, but it is unclear how cells can integrate overlapping signaling events, convert graded information into precise and discrete yet highly diverse cellular outputs, and ultimately produce a vast diversity of neurons with precise spatial organization. Perceptions of linear signaling pathways downstream of these graded morphogens have been taken predominantly from studies of simple nonmammalian systems and the ventral aspect of the spinal cord in mice. Current understanding of CNS patterning and neuronal subtype differentiation has been largely based on the assumption that changes in the concentration of extracellular morphogens can translate directly to differences in the activity of the relevant transcriptional factors and their target genes (Ashe and Briscoe, 2006). While models based on the linear response might be correct for the developmental formation of relatively simple structures, they do not appear sufficient to explain the exponential increase in both neuronal numbers and subtypes in the dorsal CNS, especially in the neocortex. Crosstalk and cooperation among overlapping mitogenic and morphogenetic pathways in mammalian CNS development are clearly necessary, but the mechanism by which fate patterning is integrated with cell-cycle progression in various progenitor domains is currently elusive.

Although Nde1-Lis1 deficiency preferentially results in the profound loss of neurons in neocortical layer 2/3 and spinal cord DI2/DI3 domains, these neurons were not completely eliminated. Moreover, all neurons in the CNS were affected by the mutation even though the ventral domains were less impaired. Thus, our data suggest a model in which cells in the developing CNS also utilize nonlinear dynamic cytoplasmic switches to coordinate cell proliferation and differentiation by controlling the mitogenic signaling threshold. Such switch-like regulation relies on the Nde1-Lis1-Brap complex to regulate the MAPK scaffold

KSR, providing proper buffer strength to set up the signaling threshold according to the local concentration of mitogens and morphogens. Such regulation must be essential for preventing excessive activation of MAPK under high local mitogenic activity in the midline and for promoting self-renewal of those progenitors localized distant from the midline and programmed to generate a large cohort of neurons. It also suggests that cells in the developmental system do not passively follow instructions from outside signals; they can also interact with the surrounding tissue and interpret the environmental cues individually to make their own fate decisions. This mechanism clearly adds an additional dimension to the linear signaling transduction model, allowing a limited number of signaling molecules to generate an exponentially increased number of cell fate outcomes. Notably, the failure in neuron generation caused by Nde1-Lis1 deficiency is most profound in structures that comprise the largest neuron populations (the neocortex and the dorsal horn). This is completely in line with the nonlinear dynamic bistable model we are proposing. Our finding supports the notion that the ability of a cell to select only a single fate out of multiple options is a trait hardwired into the cell.

Protein Scaffolds in MAPK Signaling

The MAPK cascade is one of the best-studied signal pathways responsible for interpreting a large variety of external signals and eliciting a wide range of responses in controlling cell survival, proliferation, differentiation, and migration during mammalian development. At the systems level, it has long been postulated that MAPK activation may depend on cellular context. The characteristic features of this cascade's signaling strength/amplitude, speed, and duration may determine cell fate and are believed to be regulated by protein scaffolds. These protein scaffolds may promote the assembly of key components of the cascade, provide insulation to the cascade from crosstalk with other signaling pathways, serve as docking platforms to control subcellular compartmentalization of the pathway, or temporally alter the time course of the cascade signaling (Brown and Sacks, 2009; Witzel et al., 2012). At the present, little is known about how these scaffold functions are integrated in vivo during mammalian development.

The KSR proteins are so far the best-known molecular scaffolds that tether together the three key kinases of the MAPK signaling to promote their sequential activation. The capacity of KSR to enhance MAPK signaling may be regulated by Brap, which can limit the assembly of MAPK through the inactivation of KSR. Thus, Brap has been regarded as a threshold modulator that controls the sensitivity of MAPK signaling, allows adaptations, and maintains homeostasis in a complex tissue environment. Data presented in this study are very much in line with the signaling threshold regulation of Brap. Our work showed that such threshold regulation is specifically important in CNS development, during which each progenitor cell has a unique environment and its fate choices are precisely correlated to its spatial location. Thus, our data provide a good example on how modulating the MAPK signaling threshold may play a role in developmental tissue homeostasis. Because the MAPK cascade forms the backbone of cell signaling and has an extraordinarily broad impact in cell growth and differentiation, the concept presented by this study may also be significant in the developmental morphogenesis of other tissues as well as in the proper maintenance of tissue environment, when cells are exposed to fluctuating extracellular signals. Previous studies in cell culture mainly elucidated the threshold regulation by Brap in *i*mpeding *m*itogenic signal *p*ropagation, so Brap is also known as IMP. Data from this study suggest that the function of Brap is more complex than a simple IMP. It may act more as a molecular buffer to maintain homeostasis of a cell's response to the environment. While Brap can regulate its own levels through autoubiquitination, it is unclear how the E3 ubiquitin ligase activity of Brap is regulated and whether Brap also catalyzes the ubiquitination of its associated proteins, such as Ksr. Nde1 is also known to mediate the formation of various molecular complexes and thus may play a role in Ksr stabilization. Further delineating the dynamic and spatially dependent regulation of Ksr by Brap, Lis1, and Nde1 in vivo would be essential for better understanding how the Nde1-Lis1-Brap complex modulates MAPK activity differentially in tissue morphogenesis.

Dynamic and Versatile Functions of the Nde1-Lis1-Brap Protein Interactome

Both Nde1 and Brap interact with Lis1 directly and both are scaffold proteins that interact with many other molecules besides Lis1. Similar to Lis1, Nde1 and Brap are evolutionarily conserved in eukaryotes. In mammals, Nde1 is functionally divergent from its paralog Ndel1 and shows specific expression and function in progenitors of the CNS, while Brap remains as a single ortholog. The spatially specific differential regulation of Ksr and MAPK signaling presented in this study suggests that the function of Nde1 and Brap may have evolved through increasing the number and dynamics of their interactions with other cellular proteins. Binding two or more molecules simultaneously is not only the hallmark of scaffold proteins but is also consistent with multifaceted mechanisms that allow a finite number of molecules to generate an extraordinarily larger number of outcomes owing to combinatorial interactions. The dynamic regulation by Nde1 and Brap may also be enhanced by posttranslational modifications (e.g., phosphorylation, ubiquitination) and subcellular relocalization. These may further increase the number and complexity of their protein interactions, allowing the reconfiguration of signaling networks and the rapid switches of cellular responses under variable tissue environments or stochastic noises. We believe that increasing the dynamic scaffolding capacity of Nde1 and Brap through molecular regulations at the protein level is the most effective way to gain their complex tissue-specific functions. Mice with a single mutation of Brap and Nde1 also share additional phenotypes in neural progenitor cell-cycle progression, differentiation, and survival (A.A.L. and Y.F., unpublished data). The involvement of Nde1 in other interactomes may also underlie its essential function in mammalian CNS development (S. Houlihan and Y.F., unpublished data). Therefore, it is important to further identify and study tissue-context-dependent functional interactions of Nde1 and Brap. Delineating the common and variable features of Nde1 and Brap, as well as further elucidating the dynamic rearrangements of Nde1 and Brap proteins in response to various tissue signals, would be necessary to unveil the intricacy of spatially dependent MAPK signaling and individualized cell fate regulation in the developing CNS.

EXPERIMENTAL PROCEDURES

Mouse Strains

The Nde1 and Lis1 knockout mice have been described previously (Feng and Walsh, 2004; Hirotsune et al., 1998; Pawlisz et al., 2008). The Brap knockout mouse was generated by gene targeting in mouse C57BL/6 ES cells, which was designed to insert a neo cassette flanked by loxP and FRT sites into intron 1 along with an additional loxP site into intron 3 of the murine Brap gene. Brap-targeted clones were identified by Southern blotting and injected into albino B6 blastocysts to yield germline-transmitted mice. $Brap^{+/-}$ mice were generated by crossing to the Actin-Cre line. All mice used for this study were housed and bred according to the guidelines approved by IACUC committee of Northwestern University. For timed matings, the day of vaginal plug was considered E0.5.

Immunohistochemistry and In Situ Hybridization

Immunohistochemistry (IH) and ISH analyses with mouse tissues were carried out as described previously (Feng and Walsh, 2004; Pawlisz et al., 2008). Antibodies and RNA probes used are described in the Supplemental Experimental Procedures. Quantitative analyses of IH signals were performed with ImageJ and analyzed statistically with Student's t test. Experiments were repeated with at least three litters of mouse embryos, and images from a representative experiment are shown.

Plasmids, Cell Culture, Transfection, and Immunofluorescence

Construction of Brap complementary DNA clones is described in the Supplemental Experimental Procedures. Neural progenitors from the cerebral cortex of E12.5 mouse embryos were isolated and cultured as neurospheres according to Gritti et al. (1995, 2008). Differentiation of neural progenitors was induced by plating the neurospheres to an adhesive support freshly coated with extracellular matrix gel (Sigma), withdrawing EGF and FGF-2, and adding B27 (Invitrogen). MEFs were isolated from E12 mouse embryos and cultured as described elsewhere (Feng et al., 2006). Immunofluorescence was described previously (Feng et al., 2000; Pawlisz and Feng, 2011). Antibodies used are listed in the Supplemental Experimental Procedures.

GST and immunoprecipitation was performed as described previously (Pawlisz and Feng, 2011). The binding of GST-Brap fusion proteins with His-LIS1 was detected in a buffer containing 100 mM KCl, 20 mM Tris (pH 7.6), and 0.05% Triton X-100.

Yeast two-hybrid screen and Lac-Z filter assay were performed as described elsewhere (Feng et al., 2000).

Cortical Lysates and Immunoblotting

Immunoblotting of mouse embryonic brain proteins was performed as described previously (Pawlisz and Feng, 2011). Experiments were repeated with three litters, and results from a representative experiment are shown. To detect native Brap protein from mouse embryos, flash-frozen tissue was homogenized in a detergent-free buffer containing 250 mM sucrose, 10 mM Tris (pH 7.5), and 10 mM MgCl supplemented with protease inhibitors. The homogenates were clarified by spinning in a microcentrifuge. The supernatant was analyzed by SDS-PAGE without heat denaturation.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, one table, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.devcel.2013.04.006.

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